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

No competing interests have
been declared.

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ORIGINAL RESEARCH PAPER in MICROSCOPIC FUNGI

Morphological and Molecular Characterization of Entomophthorales (Entomophthoromycota: Entomophthoromycotina) from Argentina

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Abstract

We characterized 17 insect-pathogenic entomophthoralean fungal isolates (Entomophthoromycotina: Entomophthorales) using morphological and molecular techniques. We identified four species from various insect hosts: (i) *Entomophthora planchoniana*, six specimens from aphids; (ii) *Pandora neoaphidis*, three specimens from aphids; (iii) *Zoophthora phalloides* from an aphid; and (iv) *Z. radicans*, seven specimens from insects in the orders Diptera, Hemiptera, and Lepidoptera. Analysis of ITS1 data from *E. planchoniana* showed clustering in accordance to aphid host species. *Entomophthora planchoniana* from *Macrosiphum euphorbiae* clustered together, separate from the isolate from *Myzus persicae*. The *P. neoaphidis* specimens clustered with sequences from other aphid-pathogenic *Pandora* species in GenBank. In this study, *Z. phalloides* from *Brevicoryne brassicae* and *Z. radicans* from an unidentified species of Chironomidae (Diptera) in Argentina were characterized for the first time. The present study was initiated to elucidate the taxonomy of the entomophthoralean fungi in Argentina according to their morphological and molecular characters. The presented results emphasize the significance of the combination of molecular data and information on morphology, ecology, and host range for accurate identification of entomophthoralean and allied genera.

Keywords

entomopathogens; insect pests; molecular taxonomy; host range

1. Introduction

Entomopathogenic fungi in the subphylum Entomophthoromycotina (phylum Entomophthoromycota) (Spatafora et al., 2016) are well-known virulent pathogens infecting many species of arthropod hosts. The subphylum contains over 300 species, most of which belong to the order Entomophthorales, with nearly 290 described species (Hajek et al., 2018). Most of the entomophthoralean species are obligate pathogens of insects and other arthropods (Gryganskyi et al., 2012; Hajek et al., 2018). Likewise, they are relatively host specific and, thus, pose little or no threat to non-target organisms, making these fungi ideal biological control agents against

insect pests. The host specificities of different entomophthoroid genera within the Entomophthorales are often limited to a given insect order or family; however, individual entomophthoroid species may show a higher level of specificity toward host genera or species (Humber, 2016). However, species or strains selected for commercialization as biological control agents cannot have too narrow a specificity spectrum to be economically profitable.

The identification of new strains to the species level is the first step in utilizing the full potential of fungi for specific applications (Lieckfeldt et al., 1999). Entomophthoralean species are identified on the basis of their host insect species and morphological features, primarily the size and shape of the primary conidia and the number of nuclei per conidium. However, in some cases, morphometric studies are insufficient to ascertain whether they represent a single taxon or complexes of morphologically similar species (Barta & Cagáñ, 2006). For this purpose, methods based on either biochemical reactions or DNA sequences are used. Various molecular techniques have been used in the systematics of fungi to assess interspecific variation and determine phylogenetic relationships. Up to date molecular phylogenetic studies of Entomophthoromycotina have used molecular markers such as rDNA (i.e., 18S, 28S, or the whole ribosomal operon) and protein coding regions (e.g., actin, β tubulin, and RPB2). In addition, other more variable regions (e.g., ITS) have been used to study closely related taxa (Jensen et al., 2007). Different combinations of these have been used in multiple gene analysis (Gryganskyi et al., 2013). Today, the vast majority of available genomic information within the Entomophthoromycotina is composed of partial gene and intron sequences developed for use in phylogenetic analyses. Most sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database are from the nuclear ribosomal DNA region, including the large (LSU) and small (SSU) subunits, 5.8S, and internal transcribed spacer (ITS) regions 1 and 2. Only a few sequences of entomophthoralean fungi from Argentina are available in GenBank (Jensen et al., 2009; Scorsetti et al., 2011), reflecting the paucity of information on the diversity of this fungal group in the region.

The present paper reports on the identification of *Entomophthora planchoniana* Cornu, *Pandora neoaphidis* (Remaud. & Hennebert) Humber, *Zoophthora phalloides* A. Batko, and *Zoophthora radicans* (Bref.) A. Batko from Argentinian insects and provides both morphological and molecular characterizations of these species. The fungal pathogens *Z. phalloides* and *Z. radicans* from both *Brevicoryne brassicae* L. and from an unidentified species of Chironomidae (Diptera), respectively, are recorded for first time in Argentina.

2. Material and Methods

2.1. Specimens and Morphological Characterization

Specimens were collected during sampling from crops of economic importance in 2007–2013 (Table 1). The isolates examined in this study, their host taxon, plant host, collection site, date, and GenBank accession numbers are listed in Table 1. *Zoophthora* isolates were cultivated on Sabouraud dextrose agar plus 1% yeast extract (SDAY 1%), a suitable medium used for the isolation and culture of several species of Entomophthorales (Choi et al., 2016; Feng et al., 1990; Moubasher et al., 2010; Zhou et al., 2016). *Pandora* and *Entomophthora* specimens were identified directly from infected insect hosts. Specimens were examined under a stereomicroscope and an optical microscope for the presence of rhizoids, cystidia, and/or conidia.

Fungal structures were mounted in lactophenol-aceto-orcein (LPAO) (1:1) or stained with 1% aceto-orcein plus glycerine for semipermanent mounts (as preserved material for fungarium) and measured. Fungal species were identified according to taxonomic monographs and the keys of Bałazy (1993), Keller (2007), and Humber (2012). Photographs of the primary and capilliconidia of *Z. phalloides* and *Z. radicans* from in vitro cultures were taken with an Olympus BX51 camera (Japan) at $\times 400$ magnification. Aphid host identification to the species level

was made using the keys of Blackman and Eastop (2000). Lepidopteran hosts were identified by a taxonomic specialist at the Instituto Nacional de Tecnología Agropecuaria (INTA) (see Acknowledgments).

The specimens were preserved in the Mycological Collections at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, La Plata, Argentina). The isolates were deposited in the Mycological Collections at CEPAVE and the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, New York.

2.2. Molecular Characterization: DNA Extraction and PCR Amplification

Fungus-infected insect cadavers were stored in 96% ethanol, and in vitro cultures were prepared as described by Jensen et al. (2001) until DNA extraction. Genomic DNA was extracted from infected insects (in vivo; for *Entomophthora* and *Pandora* specimens) or from in vitro cultures (for *Zoophthora* spp. specimens) via Chelex extraction (Traugott et al., 2008) or by using a DNeasy Plant Mini Kit (Qiagen, MD, USA) following the manufacturer's protocol. PCR assays were performed on three loci, the internal transcribed spacer 1 region (ITS1), the 18S gene (SSU), and the first part of the 28S gene (LSU). Because of the low quantity of DNA obtained from infected insects, PCR assays of *Entomophthora* and *Pandora* specimens were limited to the ITS1 and LSU only. Universal fungal primers or entomophthoralean-specific primers were used to avoid amplification of insect host DNA. We used the universal fungal primers ITS5 (White et al., 1990) and the single reverse primer Nu-5.8S-3' (Jensen & Eilenberg, 2001) the universal fungal primers nu-SSU-0021-58 (Gargas & DePriest, 1996) and nu-SSU-1780-38 (DePriest, 1993) for the SSU rDNA region, and nu-LSU-0018-5 (Jensen & Eilenberg, 2001) and LSU-0805 (Kjøller & Rosendahl, 2000) for LSU amplification.

The PCR conditions were: Initial denaturation for 30 seconds at 98 °C, followed by 38 cycles of denaturation for 10 seconds at 98 °C, annealing for 20 seconds at 55–60 °C (ITS1 60 °C, SSU 58 °C, LSU 55 °C), extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR reactions were carried out in 50 µL volumes, with 250 µM of each dNTP, 0.8 µM of each primer, 2.5 mM MgCl₂, 1× buffer (10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl, 0.1% Triton X-100), 1 unit of DyNzyme II (Finnzymes, Espoo, Finland), and 1 µL of extracted DNA (diluted 1:10). The size of the PCR amplification products was estimated by electrophoresis on a 1.5% agarose gel in 0.5× TBE, and the products were visualized with EZ-Vision (AMRESCO, USA). PCR products were purified using the QIAquick Purification kit (Qiagen, MD, USA) following the manufacturer's protocol. Amplicon sizes were checked by electrophoresis, and purified PCR products were sent to Macrogen (Seoul, Korea) for sequencing in both directions. The sequences obtained were submitted to NCBI GenBank (<https://www.ncbi.nlm.nih.gov/>) for gene annotation. Sequences were edited using BioEdit version 7.0.9.0 (Hall, 1999) and were used to perform a phylogenetic analysis that included some sequences of related species in the genera *Entomophthora*, *Pandora*, and *Zoophthora* available at GenBank. Sequence data for each locus were aligned with the ClustalW tool of Mega5 (Tamura et al., 2011) and trimmed, as needed, using Mesquite version 3.0.2 (Maddison & Maddison, 2009). Aligned and trimmed sequence data were submitted to TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S27276?x-access-code=5af44733057f582185683187bae29cbf&format=html>). Each dataset was analyzed using maximum parsimony (MP) in PAUP version 4.0a142 (Swofford, 2002) to determine the number of MP-informative sites. The phylogenetic analysis was carried out using the maximum likelihood (ML) method. The consensus tree was built using the latest 1,000 trees. Statistical support for the nodes was evaluated using 1,000 replicates. Phylogenetic analysis was limited to each locus; incomplete ITS1, LSU, and SSU sequence data from all specimens prevented presentation of a multilocus tree.

Table 1 List of species used in the study with information about insect and plant host taxa, location of collection, and GenBank accession numbers.

Species/strain number (CEP) or exsiccate number (CEPHe)	Insect host	Plant host	Location	Geographical coordinates		GenBank accession numbers			
				Town/city	Latitude S	Longitude W	ITS1	SSU	LSU
<i>Entomophthora planchoniana</i>									
CEPHe61	<i>Myzus persicae</i> (Sulzer)	<i>Capsicum annuum</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG252293			
CEPHe62	<i>Macrosiphum</i> sp.	<i>Rosa</i> sp.	Sunchales	30°56′00″	61°34′00″	MG252624			
CEPHe65	<i>Myzus persicae</i> (Sulzer)	<i>Solanum melongena</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG256477			
CEPHe63	<i>Macrosiphum</i> sp.	<i>Rosa</i> sp.	Sunchales	30°56′00″	61°34′00″	MG256478			
CEPHe64	<i>Macrosiphum</i> sp.	<i>Rosa</i> sp.	Sunchales	30°56′00″	61°34′00″	MG256479		MH366738	
CEPHe66	<i>Myzus persicae</i> (Sulzer)	<i>Capsicum annuum</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG256476			
<i>Pandora neoaphidis</i>									
CEPHe72	<i>Myzus persicae</i> (Sulzer)	<i>Solanum melongena</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″			MH366634	
CEPHe73	<i>Nasonovia ribisnigri</i> (Mosley)	<i>Lactuca sativa</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MH366798		MH366734	
CEPHe74	<i>Rhopalosiphum padi</i> L.	<i>Avena sativa</i> L.	Rafaela	31°12′6.62″	61°30′11.14″			MH366739	
<i>Zoophthora phalloides</i>									
CEP 687 (ARSEF 11861)	<i>Brevicoryne brassicae</i> L.	<i>Brassica oleracea</i> var. <i>botrytis</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″		MG253005		
<i>Z. radicans</i>									
CEPHe67	<i>Plutella xylostella</i> L.	<i>Brassica napus</i> L.	Ataliva	30°59′00″	61°27′00″	MG256481	MG252955	MG256485	
CEPHe68	<i>Acyrtosiphon pisum</i> (Harris)	<i>Medicago sativa</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG256480	MG252969	MG256487	
CEPHe69	<i>Brevicoryne brassicae</i> L.	<i>Brassica oleracea</i> var. <i>botrytis</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG256482	MG252968	MG256488	
CEPHe70	<i>Plutella xylostella</i> L.	<i>Brassica napus</i> L.	Rafaela	31°12′6.62″	61°30′11.14″	MG256486		MG256489	
CEPHe71	<i>Plutella xylostella</i> L.	<i>Brassica oleracea</i> var. <i>capitata</i> L.	Monte Vera	31°32′58.21″	60°41′34.74″	MG256483	MG252997	MG256490	
CEP 30 (ARSEF 6917)	<i>Tuta absoluta</i> (Meyrick)	<i>Solanum lycopersicum</i> L.	Colonia Urquiza	34°56′19.2″	58°06′3.8″	MG256484		MG256492	
CEP 320 (ARSEF 8466)	Chironomidae	unknown	La Plata	34°56′00″	57°57′00″			MG256491	

3. Results

3.1. Fungal Identification and Taxonomic Observations

Based on morphological characters, four fungal species were identified in the 17 insect specimens: *E. planchoniana*, *P. neoaphidis*, *Z. phalloides*, and *Z. radicans* (Table 1).

3.1.1. *Entomophthora planchoniana* Cornu

Bull. Soc. bot. Fr. 20: 189 (1873).

Hosts. *Myzus persicae* Sulzer and *Macrosiphum* sp. (Hemiptera: Aphididae).

Description. Primary conidia campanulate, 14.2 ± 2.5 (12.1–16.8) \times 12.1 ± 2.3 (9.8–14.5) μm , with a small apiculus and broad, nearly flattened papilla, and multinuclear (three–five nuclei). Secondary conidia were observed emerging from the primary conidia and were slightly smaller: 11.2 ± 1.72 (9.7–13.5) \times 10.5 ± 1.5 (8.7–12.2) μm (Figure 1E). Numerous thin rhizoids (Figure 1F). Cystidia and resting spores not observed. Attempts to obtain fungal isolates were not successful.

Fungarium Accession Numbers. CEPHe61, CEPHe66 (*M. persicae* from *Capsicum annuum* L.), CEPHe62, CEPHe63, CEPHe64 (*Macrosiphum* sp. from *Rosa* sp.), and CEPHe65 (*M. persicae* from *Solanum melongena* L.).

3.1.2. *Pandora neoaphidis* (Remaud. & Hennebert) Humber

Mycotaxon 34 (2): 452 (1989).

Hosts. *Nasonovia ribisnigri* (Mosley), *M. persicae*, and *Rhopalosiphum padi* L. (Hemiptera: Aphididae).

Description. Primary conidia ovoid or elongated, 23.55 ± 2.07 (19.6–27.3) \times 12.08 ± 1.55 (9.6–14.3) μm , generally conical papilla connected gently with the body of the conidia. Secondary conidia spherical or bell-shaped, 17.33 ± 1.36 (15.2–20.1) \times 13.37 ± 1.26 (11.16–15.32) μm (Figure 1G). Capilliconidia absent. Rhizoids with discoid or irregularly branched adhesive disc. Cystidia present. No resting spores were observed. Attempts to obtain fungal isolates were not successful.

Fungarium Accession Numbers. CEPHe72 (*M. persicae* from *S. melongena*), CEPHe73 (*N. ribisnigri* from *Lactuca sativa* L.), and CEPHe74 (*R. padi* from *Avena sativa* L.).

3.1.3. *Zoophthora phalloides* A. Batko

Acta Mycologica 2: 8 (1966).

Host. *Brevicoryne brassicae* (Hemiptera: Aphididae).

Description. Primary conidia elongated cylindrical to elongated oval, 33.03 ± 4.89 (24.08–42.16) \times 12.3 ± 2.52 (9.92–17.36) μm (Figure 1C). This characterization was in accordance with the original description (Batko, 1966).

The presently observed secondary conidia were capilliconidia: 18.40 ± 2.67 (12.4–22.32) \times 7.04 ± 1.22 (4.96–9.92) \times 94.63 ± 9.98 (79.36–114.08) μm (Figure 1D). Rhizoids were ramified and forming rhizomorphs. No resting spores were observed.

Culture Collection Accession Number. CEP 687 (ARSEF 11861) (*B. brassicae* from *Brassica oleracea* var. *botrytis* L.).

3.1.4. *Zoophthora radicans* (Bref.) A. Batko

Bull. Acad. Polon. Sci., Cl. II. sér. sci. biol. 12: 323 (1964).

Hosts. *Acyrtosiphon pisum* Harris, *B. brassicae* (Hemiptera: Aphididae), *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), and an unidentified species of Chironomidae (Diptera).

Description. Elongated primary conidia uninucleate, 22.63 ± 3.40 (16.36 – 25.94) \times 8.14 ± 0.75 (7.39 – 9.67), generally conical papilla demarcated with a slight protuberance from the body of the conidia (Figure 1A). Secondary conidia similar to primary ones or capilliconidia: 20.04 ± 1.83 (18.89 – 24.4) \times 7.09 ± 0.54 (6.39 – 7.93) \times 46.95 ± 9.70 (35.81 – 60.31) μm , fusiform and formed laterally on slender, capillary conidiophores arising from primary conidia (Figure 1B). Rhizoids mostly in pseudorhizomorphs, particularly abundant in the thoracical part, single or fasciculate with specialized adhesive discs. Conidiophores branched with terminal enlargement. Cystidia $3.69 \mu\text{m}$ in diameter, tapering uniformly at the base. No resting spores were observed.

Fungarium Accession Numbers. CEPHe67 (*P. xylostella* from *Brassica napus* L.), CEPHe68 (*A. pisum* from *Medicago sativa* L.), CEPHe69 (*B. brassicae* from *B. oleracea* var. *botrytis*), CEPHe70 (*P. xylostella* from *B. napus*), and CEPHe71 (*P. xylostella* from *Brassica oleracea* var. *capitata* L.).

Culture Collection Accession Numbers. CEP 30 (ARSEF 6917; *T. absoluta* from *Solanum lycopersicum* L.) and CEP 320 (ARSEF 8466; indet. Diptera from indet. host plant).

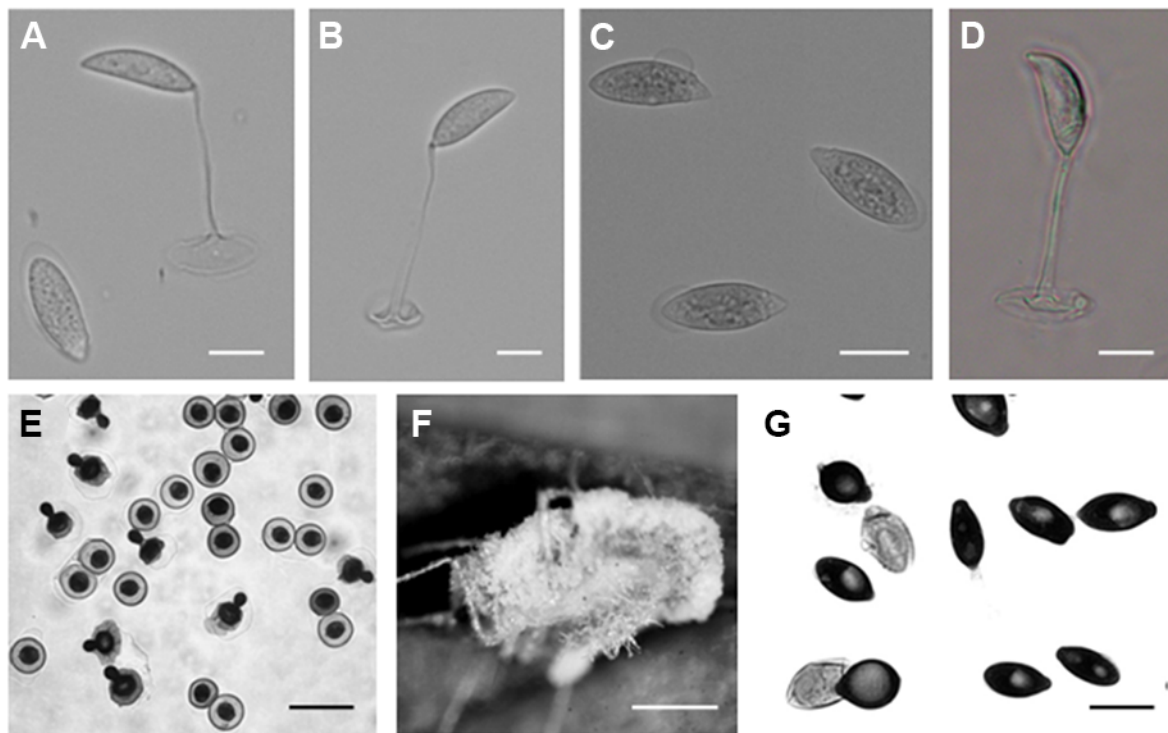


Figure 1 *Zoophthora radicans* from *Tuta absoluta* (Meyrick) primary conidium (A) and two capilliconidia each on a capilliconidiophore produced from a primary conidium (A,B). Bar: 10 μm . *Zoophthora phalloides* primary conidia (C) and capilliconidia on capilliconidiophore from primary conidium (D). Bar: 10 μm . *Entomophthora planchoniana* from *Myzus persicae* (Sulzer) primary and secondary conidia (E). Bar: 20 μm . Rhizoids of *Entomophthora planchoniana* from *Myzus persicae* (Sulzer) (F). Bar: 1 mm. *Pandora neoaphidis* from *Nasonovia ribisnigri* (Mosley) primary and secondary conidia (G). Bar: 10 μm .

3.2. Molecular Characterization: DNA Sequencing and Molecular Analyses

We successfully amplified DNA samples and obtained sequences of the ITS1 (337 bp for *Z. radicans*, 259 bp for *E. planchoniana*, and 308 bp for *P. neoaphidis*), LSU (755 bp for *Z. radicans*, 855 bp for *P. neoaphidis*, and 820 bp for *E. planchoniana*), and SSU (1,720 bp for *Z. radicans* and 1,739 bp for *Z. phalloides*). All sequences were deposited in GenBank under the accession numbers given in Table 1.

Combined with the GenBank sequences of other entomophthoralean fungi, PAUP analysis showed: ITS1 sequence data (total 733 bp) with 141 variable parsimony-uninformative and 541 parsimony-informative characters, LSU sequence data

(total 1,024 bp) with 165 variable parsimony-uninformative and 341 parsimony-informative characters, and SSU sequence data (total 1,863 bp) with 207 variable parsimony-uninformative and 54 parsimony-informative characters.

The best tree from the ML analysis of the ITS1 sequences showed that our isolates of *E. planchoniana* and *P. neoaphidis* clustered with the same respective entomophthoralean species, with a 67% bootstrap value (Figure 2). All *E. planchoniana* specimens had the same ITS1 sequence. The ITS1 sequence of *P. neoaphidis* CEPHe73 clustered with *P. neoaphidis* ARSEF 835 in another monophyletic group supported by a 99% bootstrap value. Finally, the *Z. radicans* CEPHe67, CEPHe68, CEPHe69, CEPHe70, CEPHe71, and CEP 30 sequences clustered with *Z. radicans* NW386 in another monophyletic group supported by a 100% bootstrap value. The ITS sequences of the *Z. radicans* specimens did not reveal differences between different hosts.

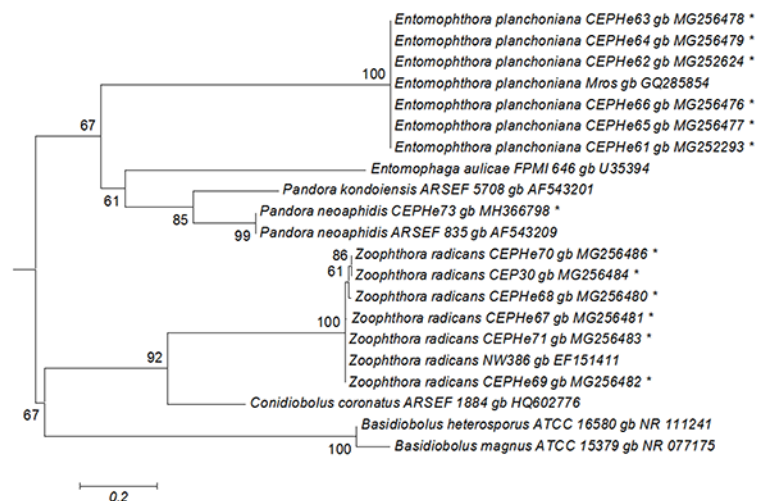


Figure 2 Phylogenetic relationships of *Zoophthora*, *Pandora*, and *Entomophthora* and related species inferred from maximum likelihood analysis of ITS1 sequences. The sequences corresponding to this work are marked with asterisks. Bootstrap values are noted above the internodes. The bar at the bottom indicates the number of substitutions per site.

In the phylogenetic analyses of the LSU rRNA, *P. neoaphidis* and *Z. radicans* were clustered in a monophyletic group with sequences from related entomophthoralean species. This grouping was supported by a 100% bootstrap value. The LSU sequences of six *Z. radicans* specimens from Diptera, Hemiptera, and Lepidoptera were identical to that of ARSEF 388, which was isolated from a dipteran in Switzerland. *Zoophthora radicans* specimen 320 had 99% similarity with the group (Figure 3). *Entomophthora planchoniana* specimen CEPHe64 clustered with *E. planchoniana* ARSEF 6252 in another monophyletic group, which was supported by a 100% bootstrap value (Figure 3).

In the ML tree from the phylogenetic analysis of the SSU rRNA sequences, *Z. radicans* CEPHe67, CEPHe68, CEPHe69, and CEPHe71 were clustered in a monophyletic group supported by a 65% bootstrap value, which includes representatives of *Z. radicans* (*Z. radicans* ARSEF 853) and *Z. lanceolata* (*Z. lanceolata* ARSEF 469) (Figure 4). Based on the SSU rRNA sequences, *Z. phalloides* CEP 687 clustered with representatives of *Z. anglica*, *Z. phalloides*, and *Z. occidentalis* in another monophyletic group supported by a 99% bootstrap value. Likewise, the sequence of *Z. phalloides* CEP 687 characterized in this work was clustered with *Z. phalloides* ARSEF 2281 in a same clade supported by a 100% bootstrap value (Figure 4).

4. Discussion

In the present study, the initial identification of fungi from various insect hosts was based mainly on the shape and size of the primary and secondary conidia and

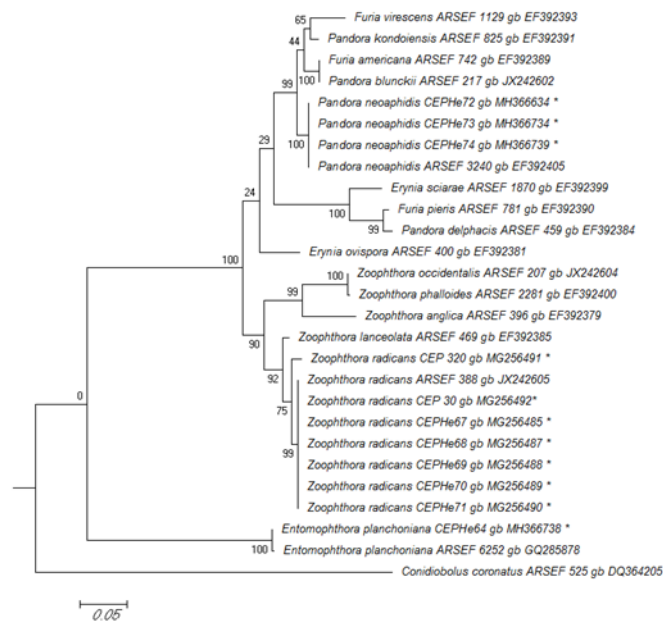


Figure 3 Phylogenetic relationships of *Zoophthora*, *Pandora*, and *Entomophthora* and related species inferred from maximum likelihood analysis of LSU rDNA sequences. The sequences corresponding to this work are marked with asterisks. Bootstrap values are noted above the internodes. The bar at the bottom indicates the number of substitutions per site.

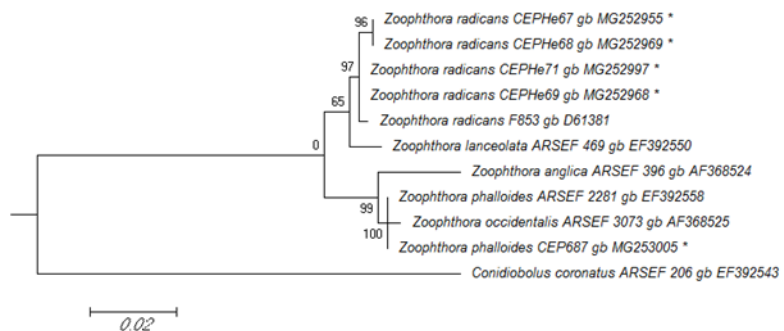


Figure 4 Phylogenetic relationships of *Zoophthora* and related species inferred from maximum likelihood analysis of SSU rDNA sequences. The sequences corresponding to this work are marked with asterisks. Bootstrap values are noted above the internodes. The bar at the bottom indicates the number of substitutions per site.

according to host affinity. These identifications were confirmed by use of molecular data, specifically the DNA sequences of the ITS1, LSU, and SSU. Based on the results obtained in this study, the morphology of the secondary conidia of *P. neoaphidis* was consistent with the original descriptions reported by Humber (1989). Our results were also consistent with observations made by Scorsetti et al. (2006). These authors previously reported *P. neoaphidis* from aphids in Argentina, with measurements of both primary and secondary conidia very similar to those observed in this study. With regards to its insect hosts, *P. neoaphidis* is a common aphid pathogen with a cosmopolitan distribution. In Argentina, it was previously recorded in 17 aphid species (López Lastra & Scorsetti, 2006; López Lastra et al., 2019; Manfrino, Hatting, et al., 2014; Manfrino et al., 2013; Scorsetti et al., 2006) and was reported by Scorsetti et al. (2006) as the most predominant pathogen of aphids in Argentina.

We identified six specimens of *Entomophthora* from aphids as *E. planchoniana*. The identity of the fungal species was determined based on insect host, number/size of conidial nuclei, and conidial size (Humber, 2012). Compared to the original

description of *E. planchoniana* (Keller, 1991), the materials observed in this study had slightly smaller conidia than those originally described. The results obtained in this study, however, were consistent with those made by López Lastra and Scorsetti (2006) and Scorsetti et al. (2006). These authors previously recorded *E. planchoniana* as a pathogen of *N. ribisnigri*, *Aphis fabae*, *Aphis gossypii*, *Myzus* sp., and *M. persicae* in Argentina and observed that the primary conidia from their specimens were slightly smaller than those described by Keller (1991). For many years, *E. chromaphidis* was considered to be a synonym for *E. planchoniana* (Gustafsson, 1965; MacLeod et al., 1976; Waterhouse & Brady, 1982) until these two species were definitely separated and justified (Humber & Feng, 1991). *Entomophthora chromaphidis* occurs in North America and Australia and is characterized as having smaller primary conidia and larger nuclei, whereas *E. planchoniana* is more widely distributed (primarily in Europe) and has larger primary conidia and smaller nuclei. In Argentina, *E. planchoniana* has been recorded as an aphid pathogen on horticultural crops (López Lastra & Scorsetti, 2006; López Lastra et al., 2019; Scorsetti et al., 2006). It has also been recorded causing epizootics in *M. persicae* on pepper and eggplant crops (Manfrino et al., 2016).

Regarding its host affinity, previous studies have already covered *Entomophthora* species. A study elucidated the host-driven divergence of species within the *E. muscae* (Cohn) Fresen. species complex and *E. planchoniana* Cornu (Jensen et al., 2009). Thomsen and Jensen (2002) showed a separation of resting spore isolates of *E. muscae* species complex at the species level, which is not possible using only morphological characters (i.e., diameter). Freimoser et al. (2001) and Jensen et al. (2009) showed that isolates originating from different specimens of the same host taxa appeared to be strongly clonal, even when they were sampled at different localities in different years. In agreement with these observations, our study showed only minor differences in *E. planchoniana* specimens from several different aphid species based on ITS1 sequences.

In this study, the primary and secondary conidia of *Zoophthora radicans* specimens were slightly bigger than the original description reported by Keller (1991). Our results are consistent with reports by López Lastra and Scorsetti (2006) regarding the measurements of the primary conidia of *Z. radicans* from *T. absoluta*, although we observed bigger capilliconidia. Scorsetti et al. (2006) recorded *Z. radicans* from six aphid species, and our measurements of the primary and secondary conidia of this species are consistent with their data. Additionally, these authors recorded *Zoophthora* sp. from only one aphid species and found that its morphological characteristics clearly differed from those of *Z. radicans*. In this study, measurements of *Zoophthora* from *B. brassicae* were different from those of *Z. radicans*, with primary and secondary conidia of greater values. Based on the original description by Batko (1966), this species corresponds to *Z. phalloides*. Glare et al. (1987) studied different criteria based on morphological, physiological, and biochemical characters in order to reliably differentiate *Z. phalloides* from *Z. radicans*. This allowed the division of isolates broadly defined as *Z. radicans* and *Z. phalloides* using shape and spore dimensions, host specificity, growth in vitro, and analyses of isoenzymes and fatty acid composition (Glare et al., 1987). Balazy (1993) conducted thorough studies on the morphology, biology, and particularly host specificity of the genus *Zoophthora*, which enabled him to distinguish and describe seven new species morphologically similar to *Z. radicans*. *Zoophthora phalloides* is very similar morphologically to other aphid pathogenic members of the *Zoophthora* spp. complex. *Zoophthora phalloides* was first described as a pathogen of aphids (Hemiptera) in Poland by Batko (1966) and has since been recorded in North America, Britain, Denmark, France, Switzerland, Israel, Korea, New Zealand, and occasionally in Australia (e.g., Barta & Cagán, 2005; Nielsen et al., 2001; Papierok et al., 2016; Yoon et al., 1998), thus indicating a worldwide distribution.

Zoophthora radicans has the broadest host range reported for any species of the Entomophthoraceae. This observation has led some authors to suggest that it is a complex of several species differing in their pathogenicity to different hosts (Balazy, 1986; Glare, 1988; Humber, 1983; McGuire et al., 1987). It has

been collected worldwide from hosts in the insect orders Lepidoptera, Diptera, Coleoptera, Orthoptera, Thysanoptera, Hemiptera, and Hymenoptera (Glare & Milner, 1991; Keller, 1991; Milner & Soper, 1981; Papierok et al., 1984; Soper & Ward, 1981). In Argentina, *Z. radicans* has been reported as a pathogen of several insect species (Manfrino, Hatting, et al., 2014; Manfrino et al., 2013; Manfrino, Zumoffen, et al., 2014; Scorsetti et al., 2006), but there were no records of dipteran hosts until this study. Milner and Mahon (1985) identified *Z. radicans* in other species of Diptera (*Damaromyia* sp., Sciaridae, Psychodidae) in Australia. Our results revealed that the ITS1, LSU, and SSU sequences of *Z. radicans* specimens from Diptera, Lepidoptera, and Hemiptera show few differences. In analyses of LSU sequences, all specimens clustered as *Z. radicans*, with a slight difference for the dipteran isolate, in agreement with reports of its wide host range.

For species included within a species complex, molecular characters are critical data supplementing analysis at different taxa. In this study, species of *Zoophthora* in Argentina were characterized by molecular tools for the first time. Molecular analyses based on SSU rDNA sequences confirmed the identity of *Z. phalloides*, with the Argentinean strain grouped with *Z. phalloides* strain ARSEF 2281 (gb EF392558) with a 100% bootstrap value. Likewise, with regards to host affinity, the *Zoophthora* aphid-pathogenic isolate CEP 687 was clustered with isolate ARSEF 2281 (*Z. phalloides*), which was originally isolated from *B. brassicae*, in accordance with the host aphid species of our native strain (Figure 4).

This study reports the first record of *Z. phalloides* as a pathogen of *B. brassicae* and *Z. radicans* as a pathogen of an unidentified species of Chironomidae (Diptera) in Argentina. The identification and establishment of in vitro cultures of these fungal species would allow further studies determining their potential as microbial biological control agents for the management of insect pests of agricultural crops. Of the four fungi, *Zoophthora* spp. and *Pandora neoaphidis* can be grown on SDAY, but *Zoophthora* spp. are easier to cultivate than *P. neoaphidis* on this medium. Other entomophthoralean species included in the genus *Entomophthora* require complex and highly nutritious culture media, which is considered a sign of high specialization and close adaptation to the host (Humber, 1994). Based on these observations, *Zoophthora* strains would have greater potential to be used as targeted microbial control agents.

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